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In Vitro Study on Assessment of Petrol, Kerosene and Diesel Degrading Potential of Indigenous Fungal Isolates From Different Petroleum Product Effected Soils

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Abstract- **Application of micro organisms for effective removal of hydrocarbon contamination from soil has been considered by several workers since decontamination of polluted soil by other methods leads to production of toxic compounds and these techniques are non-economic also. Therefore, in the present study, soil samples from three highly aged petroleum contaminated sites were studied for assessment of their petrol, kerosene and diesel degrading potential by fungal isolates. Twenty native fungi species belonging to eight fungal genera were isolated from different petroleum contaminated soil samples. The identified fungal genera included *Aspergillus sp., Fusarium sp., Penicillium sp., Rhizopus sp., Candida sp., Cladosporium sp., Galactomyce sp. and Sympodiomycopsis sp.* based on 18S rRNA identification. The closely related sequences were aligned to construct a phylogenetic tree for these fungal isolates and were deposited in NCBI, GenBank under different accession numbers. Biodegradation ability of all isolates was confirmed by shake flask culture and vapour phase transfer method. The results showed that indigenous fungal isolates *Aspergillus sp., Penicillium sp. and Rhizopus sp.* displayed highest capability of biodegradation of the petroleum products. Hence, these fungal species can be effectively utilized for the degradation of petrol, kerosene and diesel.**

Key words- **Petroleum contaminated soils, Fungal isolates, 18S rRNA, Biodegradation ability, Phylogenetic analysis.**

I. INTRODUCTION

Petroleum like all fossil fuels primarily consists of a complex mixture of molecules called hydrocarbons. In large concentrations, the hydrocarbon molecules that make up crude oil and petroleum products are highly toxic to many organisms, including humans [1]. The dominance of petroleum products in the world economy creates the conditions for distributing large amounts of these toxins into populated areas and ecosystems around the globe [2]. The most rational way of decontamination of the environment loaded with petroleum derivatives is an application of methods based mainly on metabolic activity of microorganisms [3]. However, single cultures of fungi have been found to be better than mixed cultures [4] and more recently, fungi have been found to be better degraders of petroleum than traditional bioremediation techniques including bacteria [5].

Although, hydrocarbon degraders may be expected to be readily isolated from a petroleum oil-associated environment, the same degree of expectation may be anticipated for microorganisms isolated from a totally unrelated environment [6]. The source of fungi for the efficient biodegradation of petroleum products has become the major concern however, the source of fungi is selected from polluted soils or waters (their natural environments), rhizoplanes, food or crops and root tubers contaminated by petroleum products.

Recently, many researchers studied the role of fungi in biodegradation process of petroleum products and the most common fungi which have been recorded as biodegraders belongs to following genera: *Alternaria, Aspergillus, Candida, Cephalosporium, Cladosporium, Fusarium, Geotrichum, Gliocladium, Mucor, Paecilomyces, Penicillium, Pleurotus,*

Polyporus, Rhizopus, Rhodotolura, Saccharomyces, Talaromyces, Torulopsis etc [7-9] .Therefore, in the present investigation an assessment of petrol, kerosene and diesel degrading potential of indigenous fungal isolates from different petroleum product contaminated soil sites has been carried out.

II. MATERIALS AND METHODS

Surface soil samples at 0-15 cm depth, weight of 400 g were collected from three different sites which were contaminated with petrol, kerosene and diesel separately along with non-contaminated garden soil. Samples were made from 3-4 random locations per site, mixed and transferred into sterile bottles using sterile spatula for microbiological quality determination and stored in ice box to avoid contamination. In the lab, stones and other unwanted soil debris were removed by using 2.5 mm sieve, one gram of each sorted soil sample was homogenously mixed with 1 drop (0.1 ml) of Tween 80. 1.0 g of homogenized, 2 mm sieved soil sample was aseptically transferred, using a flame-sterilized steel spatula, into a sterile test tube containing 9.0 mL of sterile distilled water. This gave 10^{-1} dilution and subsequently, three-fold (10^{-3}) serial solutions were prepared from the 10^{-1} dilution. 1 ml of dilution was poured on Potato Dextrose Agar (PDA) plates and Soubraurd Dextrose Agar (SDA) plates, Streptomycin (500 mg/l) as antibiotic inhibit bacterial growth was added to the media after sterilization process [10-11]. Afterwards, the plates were incubated at a temperature of 28 – 31^{o}C for 48hours or more depending on the rate of growth. To obtain pure cultures of the fungal isolates, fungal cultures were aseptically subcultured into fresh PDA and SDA plates and incubated until the fungus begins to sporulate followed by subsequent sub culturing to get pure cultures consisting of only one type of fungus isolates. A part of the pure culture was then aseptically transferred into sterile agar slants which had previously been prepared in sterile sugar tubes. The sugar tubes are then incubated till full growth of the fungus and they then serve as stock cultures.

Bushnell-Haas broth medium was used for the primary screening test which composed of: MgSO4 (0.2 g/l), CaCl2 (0.02 g/l), KH2PO4 (1 g/l), K2HPO4 (1 g/l), FeCl2 (0.05 g/l) and NH4NO3 (1 g/l). Tween 80 (0.1%), redox reagent (2% 2, 6-dichlorophenol indophenols) and petroleum products (1% of each Kerosene, Petrol, Diesel) were administered into the broth [12-13]. For vapour phase transfer method, mineral salt medium (MSM) was prepared according to modified Mills et al. (1978). The composition of the medium was NaCl (10.0 g), $MgSO_4.7H_2O$ (0.42 g), KCl (0.29 g), KH_2PO_4 (0.83 g), Na_2HPO_4 (1.25 g), $NaNO_3$ (0.42 g), agar (20 g), distilled water (1 L) and pH of 7.2.

III. IDENTIFICATION OF FUNGAL ISOLATES

Fungal genera were identified according to morphological characters [14]. The inoculated plates were identified on the basis of cultural (colour and colonial appearance of fungal colony) and morphological characteristics. Species were identified by using DNA sequence method. A suitable mass of inoculum of fungal isolate was prepared with carefully removing the upper surface of the isolate without agar medium. The DNA extraction technique used to remove inhibitory materials, i.e. polysaccharides, proteins, mineral salts, etc., which limit the sensitivity of the different reactions in which isolated DNA is applied [12], [15]. Genomic DNA was isolated from the fungal sample using Chromous fungal genomic DNA isolation kit following manufacturer's protocol (Chromous Biotech., Banglore, India). Approximately 100 mg of fungal hyphae were scraped off from petridishes and transferred to bead tubes provided in the kit. Mechanical lysis was enhanced using a Talboys High Throughput Homogenizer (Troemner, Thorofare, NJ, USA) at 1600 rpm for 3 minutes. DNA extracts were assessed using a Nano-drop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Fungal 18S rRNA genes were PCR amplified using 5'- GTAGTCATATGCTTGTCTC -3'and 5'- GAAACCTTGTTACGACTT -3' primers. Reactions were performed in 100 µl volumes containing 4 µl dNTPS, 400ng of each primer, 10X Taq DNA Polymerase Assay Buffer 10 µl, 1 µl of Taq DNA Polymerase enzyme, and 1 µl template DNA. Thermo cycling conditions consisted of an initial de-naturation stage of 94°C for 5 minutes followed by 35

cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 min, and a final stage of 72°C for 5 minutes.

PCR products obtained were Gel eluted using Chromous Gel extraction kit and sent for sequencing on an ABI 3500 XL Genetic Analyzer (Applied Biosystems Inc., Foster City CA, USA). The basic local alignment search tool-BLAST was used to classify and identify closely related fungal sequences [12].

Nucleotide Sequence Accession Numbers

The nucleotide sequences reported in this paper have been submitted to the NCBI, GenBank database under the following accession numbers KC545836 to KC545875 (Table I).

Primary step for confirming biodegradation potential of fungal isolates (Shaking flask method).

The biodegradability of isolated fungi was verified using the modified technique based on the redox indicator 2, 6-dichlorophenol indophenol (DCPIP) [13], [16]. Two plugs from 7 days old fungal isolates (1 cm^2 for each plug) were picked from the peripheral area of petri-dish and transferred carefully into 50 ml Bacto Bushnell-Haas broth medium using 250 ml conical flask. 0.1% (v/v) Tween 80 and 1% (v/v) petrol, kerosene and diesel and 0.008 mg/50 ml of redox indicator as a powder were added to the Bacto Bushnell-Haas broth medium. All flasks incubated in room temperature using a shaker with 180 rpm for seven days. Five mL of the aliquots were collected daily from each flask and the absorbance was noted on spectrophotometer at 600 nm wavelength. Change in color of inoculated media in the flasks from deep blue to colorless indicates the ability of fungi to biodegrade petroleum products like petrol, kerosene and diesel.

Final confirmation for biodegradation potential of fungal isolates (Vapor phase transfer method)

Fungal isolates showing better performance (fastest colour change) in primary step were further tested by vapor phase transfer petroleum products utilization test [13], [17]. This test was carried out for the confirmatory identification of actual petroleum products-utilizing moulds. For petroleum product utilization test, mineral salt medium (MSM) was prepared according to modified media of [18] however, petroleum products were made available to the inoculants via vapor phase transfer instead of media. Putative -utilizing fungal isolates in first step of confirmation were streaked on plates of agar medium (one isolate per plate). Inner side of petridish was covered with a sterile filter paper (Whatman No. 1) saturated with filter-sterilized petrol, kerosene and diesel. The main aim was to supply petroleum products (source of hydrocarbons) as sole source of carbon and energy for the growth of the fungi on the mineral salts agar medium surface through vapor phase transfer. All the plates were inverted and incubated at 27°C for 7-14 days [19]. Colonial development of different fungi appearing on the mineral salts agar medium was confirmed as petroleum products utilizers.

IV. RESULTS

Results revealed that 20 fungal strains belonging to a total of eight genera were isolated from the four different soils during the present investigation. These include *Aspergillus (A.* terreus, A. versicolor, *A. niger,A.fumigatus)*; *Fusarium* oxysporum.; *Cladosporium* bruhnei, *Penicillium (p.* janthinellum, *P.* decumbens *)* , *Candida tropicalis, Galactomyce geotrichum., Sympodiomycopsis paphiopedili* and *Rhizopus* oryzae (Table I).

Biodegradation potential of Fungal isolates

During primary step for confirming biodegradation potentials of fungal isolates, out of the total identified eight genera, three genera such as *Aspergillus* sp.*; Candida* sp.; and *Cladosporium* sp. were found Kerosene-utilizers. Two genera viz.: *Fusarium* sp. and *Galactomyce sp*.; were effective petrol degraders and four genera viz., *Rhizopus* sp.; *Aspergillus sp.; Penicillium sp.; and Sympodiomycopsis sp*. showed efficiency for diesel degradation (Fig. 1).These isolates produced a colour change in the Bacto

Bushnell-Haas broth medium. The absorbance of broth medium changed according to degradation capacity rate and extent in each flask. Almost total colour change (blue to colourless) was also observed in some flasks while in other flasks colour changes up to some extent. Among the better performing seven isolates , *Penicillium janthinellum, Aspergillus terreus, Rhizopus oryzae , and Penicillium decumbens* displayed the fastest onset colour change (decrease in absorbance of broth medium) and hence, highest capability of biodegradation (Fig. 2). However, there is no colour change in control which refers the media without fungal inoculums. These figures show the decrease in the absorbance of the Bacto Bushnell Haas broth medium after the fungal inoculation.

For confirmatory identification of actual petroleum products-utilizing moulds, all fungal isolates which were taken in primary step were further tested by vapour phase transfer petrol, kerosene and diesel utilization test. After 10 days of incubation, the six fungal isolates i.e., *Aspergillus terreus, Fusarium oxysporum, Rhizopus oryzae, Aspergillus niger, Penicillium decumbens, Penicillium janthinellum* show better growth during petroleum products treatment by vapour phase transfer method. These further prove petrol, kerosene and diesel biodegradation potentials by these fungal isolates (Fig. 3).

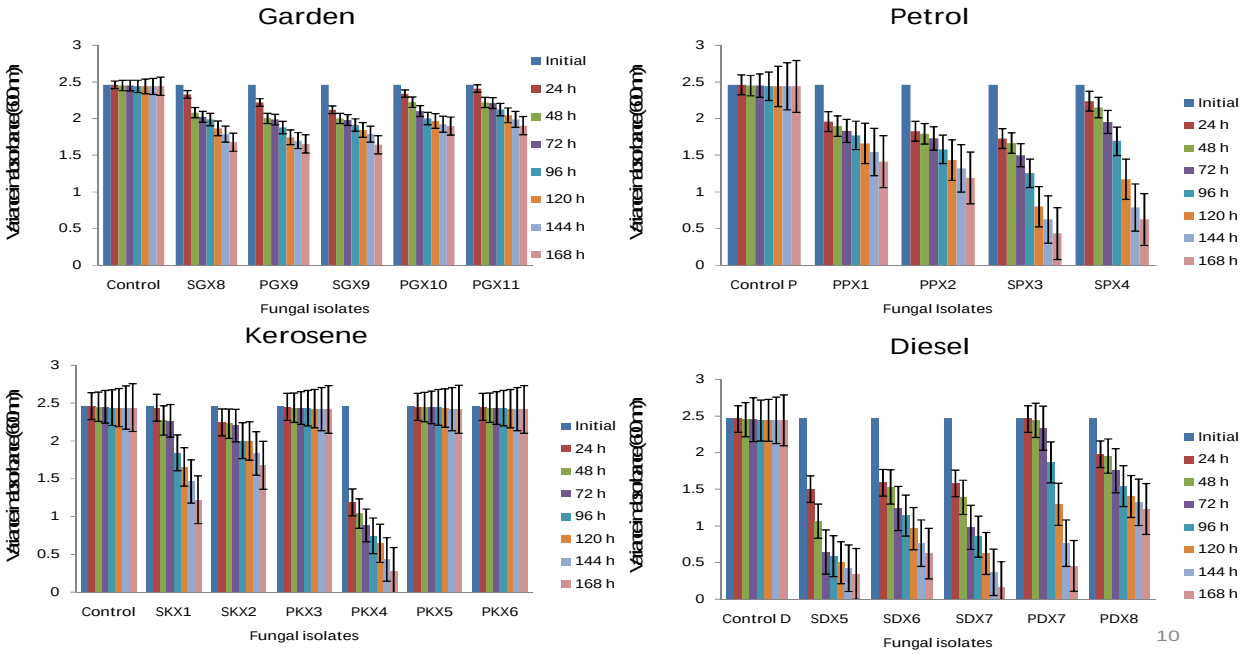

Fig. 1 Variation in the absorbance of Bacto Bushnell Hass broth medium by different fungal isolates (abbreviations in this fig. expanded in Table I)

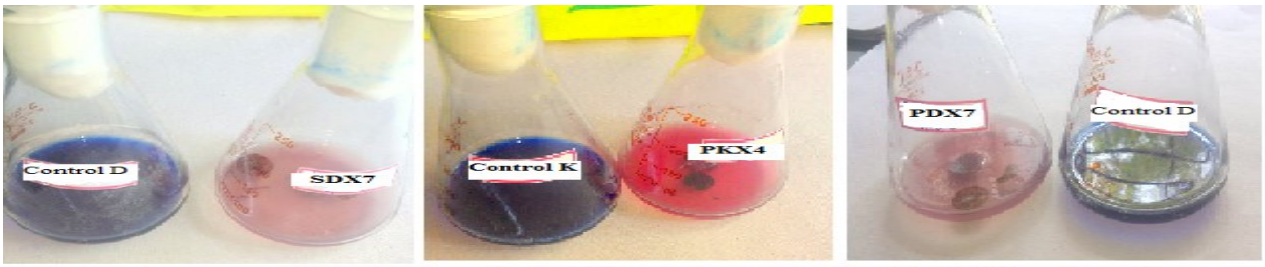

Fig. 2 Fungal growth observed in vapour phase transfer petrol, kerosene and diesel utilization test after 10 days of incubation

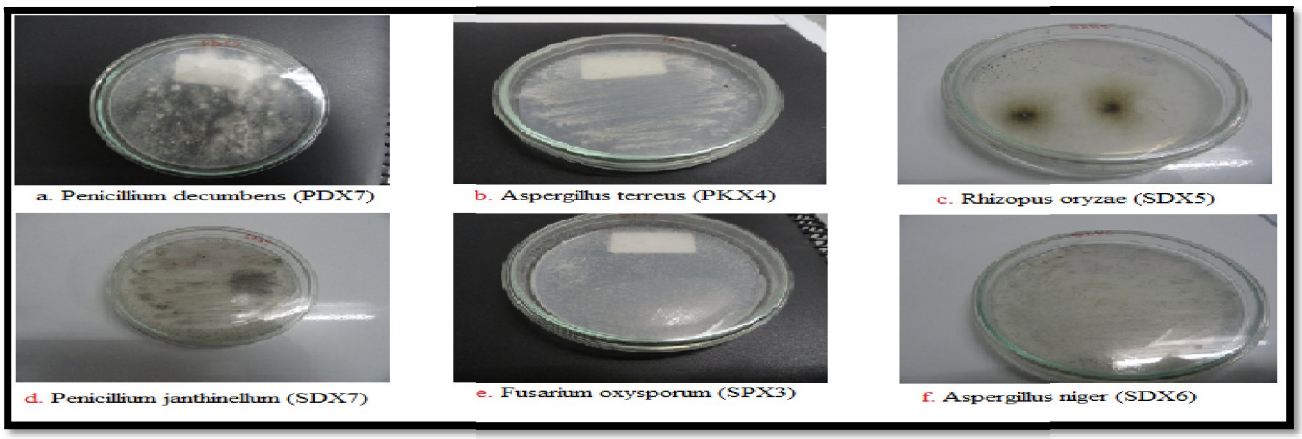

Fig. 3 Biodegradation ability of Petroleum products by fungal strains (Comparison between control and the three better performing fungal strains)

V. DISCUSSION

The partial 18S rRNA gene sequence described in this paper revealed a high level of conservation and relatively late onset of divergence in the fungal isolates from contaminated and non-contaminated soils. It appears that the morphological functional and petroleum product adaption of the fungal isolates is not mirrored by the molecular evolution of these organisms which is supported by the findings of [20] while studying rRNA sequence and evolutionary relationships among toxic and nontoxic cyanobacteria.

During primary step for confirming biodegradation potentials of fungal isolates, the ability of these isolates to produce a colour change in the Bacto Bushnell-Haas broth medium is presumably due to the reduction of the indicator by the oxidized products of hydrocarbon degradation. The total colour change (blue to colourless) supports the fact that the isolates are potential hydrocarbon oxidizers. Among better performing seven isolates which produced significant colour change species *Penicillium janthinellum*, *Aspergillus terreus* ,*Rhizopus oryzae and Penicillium decumbens* displayed the fastest onset color disappearance (decrease in absorbance of broth medium) and hence, highest capability of biodegradation. The high rate of petroleum products (hydrocarbon) degradation by the three fungi could emanate from their massive growth and enzyme production responses during their growth phases. This could be supported by the findings of [21], who suggested that extracellular ligninolytic enzymes of white rot fungi are produced in response to their growth phases. Besides, the utilization of 0.1% of Tween 80 during the assay and the implication of these three organisms in hydrocarbon degradation from our results is similar to the findings of [22]. In vapour phase transfer petroleum product utilization test after 10 days of incubation for confirmatory

identification of actual petroleum products-utilizing moulds, the six fungal isolates i.e., *Aspergillus terreus, Fusarium oxysporum, Rhizopus oryzae, Aspergillus niger, Penicillium decumbens and Penicillium janthinellum* showed better growth during petroleum products treatment by vapour phase transfer method.,which further confirming petrol, kerosene and diesel utilization and biodegradation potentials of these fungal isolates, and corroborated with the findings of [13] while studying the diesel degrading potential of fungal isolates from sludge contaminated soils of petroleum refinery, Haryana, India.

An interesting findings generated in this work show that an increase in rates of fungal growth in the media containing petroleum products as compared with media without petroleum products, this might be due to the fact that the fungi use petroleum products as a substrate for their survival growth and using extra cellular enzymes to break down the recalcitrant hydrocarbon molecules, by dismantling the long chains of hydrogen and carbon, thereby, converting petroleum into simpler forms or products that can be absorbed for the growth, development and nutrition of the fungi .

VI. CONCLUSION

In this study, we observed that higher biodegradation efficiency was encountered by *Penicillium janthinellum, Aspergillus terreus, Rhizopus oryzae and Penicillium decumbens,* providing these fungi to be better petroleum product degraders. Thus, they can be effectively utilized for the degradation of petrol, kerosene and diesel for biodegradation of oil polluted or contaminated soils especially those located within the vicinity of the petroleum processing and disposal sites.

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Table I

Fungal taxa isolated from different petroleum product contaminated and non-contaminated soils identified by length of DNA sequences by BLAST analysis

SOIl	Sequence ID	Length	*TOP BLAST*	% Similarity	Class	Order	Family	NCBI GenBank accessions numbers.	
								Forward	Reverse
GARDEN	SGX8	2713	*Penicillium janthinellum*	99%	Eurotiomycetes	Eurotiales;	Trichocomaceae	KC545846	KC545847
	PGX9	1687	*Aspergillus niger strain* HKS11	99%	Eurotiomycetes	Eurotiales	Trichocomaceae	KC545848	KC545849
	SGX9	1714	*Fusarium oxysporum*	100%	Sordariomycetes	Hypocreales	Nectriaceae	KC545850	KC545851
	PGX10	1733	*Aspergillus terreus*	99%	Eurotiomycetes	Eurotiales	Trichocomaceae	KC545852	KC545853
	PGX11	1733	*Aspergillus versicolor*	99%	Eurotiomycetes	Eurotiales	Trichocomaceae	KC545854	KC545855
KEROSENE	SKX1	1704	*Candida tropicalis*	99%	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	KC545856	KC545857
	SKX2	1687	*Aspergillus niger strain* HKS11	100%	Eurotiomycetes	Eurotiales	Trichocomaceae	KC545858	KC545859
	PKX3	1747	*Cladosporium bruhnei strain* USN 11	99%	Dothideomycetes	Capnodiales	Davidiellaceae .	KC545860	KC545861
	PKX4	1729	*Aspergillus terreus*	100%	Eurotiomycetes	Eurotiales	Trichocomaceae	KC545862	KC545863
	PKX5	1714	*Fusarium oxysporum* isolate K9	100%	Sordariomycetes	Hypocreales	Nectriaceae	KC545864	KC545865
	PKX6	1699	*Aspergillus versicolor strain HDJZ-*ZWM-16	100%	Eurotiomycetes	Eurotiales	Trichocomaceae	KC545866	KC545867
GASOLINE	PPX1	1687	*Aspergillus niger strain* HKS11	99%	Eurotiomycetes	Eurotiales	Trichocomaceae	KC545868	KC545869
	PPX2	1747	*Cladosporium bruhnei strain* USN 11	100%	Dothideomycetes	Capnodiales	Davidiellaceae .	KC545870	KC545871
	SPX3	1767	*Fusarium oxysporum*	100%	Sordariomycetes	Hypocreales	Nectriaceae	KC545872	KC545873
	SPX4	1668	*Galactomyceae geotrichum* strain SK15	100%	Saccharomycetes	Saccharomycetales	Dipodascaceae	KC545874	KC545875
DIESEL	SDX5	1757	*Rhizopus oryzae* 99-133	99%	Phycomycetes	Mucorales	Mucoraceae	KC545836	KC545837
	SDX6	1771	*Aspergillus niger strain* YM33182	98%	Eurotiomycetes	Eurotiales	Trichocomaceae	KC545838	KC545839
	PDX7	1769	*Penicillium decumbens strain* ML-017	99%	Eurotiomycetes	Eurotiales	Trichocomaceae	KC545840	KC545841
	SDX7	2713	*Penicillium janthinellum*	100%	Eurotiomycetes	Eurotiales	Trichocomaceae	KC545842	KC545843
	PDX8	1786	*Sympodiomycopsis paphiopedili* AFTOL-ID 1772	99%	Exobasidiomycetes	Microstromatales incertae sedis	Sympodiomycopsis	KC545844	KC545845